ANTICANCER ACTIVITY OF SILVER NANOPARTICLES SYNTHESISED BY *Catharanthus roseus* AQUEOUS EXTRACT

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ABSTRAK

Nanopartikel perak (AgNPs) semakin banyak digunakan dalam aplikasi bioperubatan kerana aktiviti antimikrob yang luar biasa mereka. Walau bagaimanapun, aktiviti anti-kanser daripada AgNPs kekal akan disiasat. Kajian ini bertujuan untuk menilai aktiviti *in vitro* anti-kanser AgNPs terhadap sel kanser Jurkat (T-sel leukemik) dan HT29 (kolorektal kanser). AgNPs biogenik telah disintesis menggunakan ekstrak akueus daun *Catharanthus roseus* (*C. roseus*). Aktiviti proliferatif *C. roseus*-AgNPs telah dikira menggunakan MTS *assay*, diikuti dengan pengesanan apoptosis menggunakan annexin-FITC/propidium iodida *assay* dan aktiviti kitaran sel menggunakan kitaran sel *assay*. Nilai IC_{50} *C. roseus*-AgNPs terhadap sel Jurkat adalah dalam julat 1.96 hingga 626 μg/mL (median = 5.87 μg/mL) pada pelbagai masa inkubasi. Sementara itu, nilai IC_{50} terhadap sel HT29 adalah dalam julat 12.99 hingga 13.39 μg/mL (median = 13.19 μg/mL) pada pelbagai masa inkubasi. Sebagai perbandingan, nilai IC_{50} ekstrak akueus *C. roseus* pada sel-sel Jurkat berada dalam julat 23.43 hingga 700.00 μg/mL (median = 361.72 μg/mL) manakala nilai IC_{50} terhadap sel HT29 adalah dalam julat 191.47 hingga 888.00 μg/mL (median = 419.26 μg/mL) pada pelbagai masa inkubasi. Peratusan yang tinggi bagi kedua-dua apoptotik awal dan lewat dikesan terhadap sel Jurkat berbanding terhadap sel HT29 pada pelbagai masa inkubasi, sebagai tindak balas kepada *C. roseus*-AgNPs. *C. roseus*-AgNPs dan ekstrak akueus *C. roseus* telah merencat fasa S terhadap sel Jurkat pada setiap inkubasi manakala pada HT29, *C. roseus*-AgNPs dan ekstrak akueus *C. roseus* telah merencat sama ada fasa G_0/G_1 dan S

di pelbagai inkubasi. Hasil kajian menunjukkan*C. roseus*-AgNPs mempunyai potensi untuk digunakan sebagai agen anti-kanser, terutama pada leukemia dan kanser usus.

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ABSTRACT

Silver nanoparticles (AgNPs) are increasingly used in biomedical applications because of their remarkable antimicrobial activity. However, the anticancer activity of AgNPs remain to be investigated. The present study was aimed to evaluate *in vitro* anticancer activity of AgNPs on Jurkat (leukemic T-cells) and HT29 (colorectal cancer) cancer cell lines. The biogenic AgNPs were synthesised using *Catharanthus roseus*(*C. roseus*) leaf aqueous extract. The proliferative activity of *C. roseus*-AgNPs was measured using MTS assay, followed by the detection of apoptosis using annexin-FITC/propidium iodide assay and cell cycle activity using cell cycle assay. The IC_{50} values of *C. roseus*-AgNPs on Jurkat cells were in the range of 1.96 to 6.26 µg/mL (median = 5.87 µg/mL) at various incubation times. Meanwhile, the IC_{50} values on HT29 cells were in the range of 12.99 to 13.39 µg/mL (median = 13.19 µg/mL) at various incubation times. In comparison, the IC_{50} values of *C. roseus* aqueous extract on Jurkat cells were in the range of 23.43 to 700.00 µg/mL (median = 361.72 µg/mL) while the IC_{50} values on HT29 cells were in the range of 191.47 to 888.00 µg/mL (median = 419.26 µg/mL) at various incubation times. Higher percentages of both early and late apoptotic cells were detected with for Jurkat cells compared to HT29 cells at various incubation times, in response to either *C. roseus*-AgNPs or *C. roseus* aqueous extract. *C. roseus*-AgNPs and *C. roseus* aqueous extract have arrested S phase in Jurkat cells at all incubations while in HT29, the *C. roseus*-AgNPs and *C. roseus* aqueous extract have arrested either G_0/G_1 and S phase at various incubations. The findings indicate that the *C. roseus*-AgNPs have a potential to be used as anticancer agents, particularly on leukemia and colon cancer.

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LIST OF SYMBOLS AND ABBREVIATIONS

˚C	=	degree Celcius
%	=	percentage
µg/mL	=	Microgram per milliliter
h	=	hour
g	=	gram
min	=	minute
ml	=	milliliter
mM	=	miliMolar
nm	=	nanometer
g	=	Gravity force
w/v	=	weight per volume
v/v	=	volume per volume
AgNPs	=	Silver nanoparticles
IC_{50}	=	Inhibitory Concentration at 50%

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nano oncology is the new field of research knowledge that has potential to be used in the detection, target and treatment of the cancer (Jacob et al., 2013). Nanoscale treatment is advantageous as compared to the micro-sized anticancer drugs since it is more stable and specific, long shelf life, and required only limited dose frequency (Jacob et al., 2013, Rajesh and James, 2009). This is due to their unique characteristics such as large surface area per volume ratio, porosity, solubility, increased bioavailability and different structural properties. The small sized nanoparticles also make it able to pass through the cellular barriers and strongly interacts with functional biomolecules (Gnanasekar et al., 2014). These characteristics make them as a favourable choice to overcome the limitations of conventional cancer therapy, for instance less effectiveness of the chemotherapeutic drugs to reach the targeted sites (Boca et al., 2011, Shakti et al., 2013).

A report provided by Bhattachryya et al. (2011) has summarised the list of nanoparticle-derived drugs with their product names in different phase of clinical development including iron oxide nanoparticles (Combidex) for cancer imaging, TNF-a bound PEGlyated colloidal gold particles (Aurimmune) for solid tumors and nano-sized calcium phosphate (Biovant) for vaccine component whereas albumin-bound paclitaxel particles (Abraxane) have been approved by Food and Drug Administration (FDA) to be applied for the treatment of non-small cell lung carcinoma and breast cancer. The

mechanisms of cytotoxicity for each type of nanoparticles were found to be different and thus, the application differs from each other.

Among various types of nanomaterials, silver are advantageous in regards with their unique properties, that include conductivity, stability, catalytic and antibacterial property (Jeyaraj et al., 2013). Silver nanoparticles (AgNPs) can be defined as nanoscale metals sized within 1 to 100 nm (Sukumaran and Eldho, 2012) and have been widely used in biomedical applications including as anti-inflammatory, antioxidant, antimicrobial and antibacterial agents towards many bacterial genera include *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, (Saranyaadevi et al., 2014). It has been widely used in medical devices, catheters, dental fillers, wound dressing and also for therapeutic purposes (Sangiliyandi et al., 2013b). However, the potential applications of these silver nanoparticles as anticancer agents are still new and remain to be investigated.

Previous studies have documented the cytotoxicity effects of AgNPs on various cancer cell lines with promising results. For example, silver nanoparticles have been proven to be biocompatible to healthy cells and has inhibitory effects on the proliferation of various cancer cell lines (Boca et al., 2011) including human glioblastoma cells (AshaRani et al., 2009), Dalton's lymphoma ascites (DLA) cell lines (Muthu et al., 2010, Jacob et al., 2013), human cervical carcinoma cell lines (Karunamoorthy et al., 2014), breast carcinoma MCF-7 cell lines (Ramy and Hashem, 2014, Gnanasekar et al., 2014), HeLa cell lines (Panchanathan et al., 2013), MDA-MB-231 cell lines (Sangiliyandi et al., 2013a) and human lung cancer A549 cell lines (Renu

et al., 2013, Balaji et al., 2014). These preliminary data suggests that AgNPs have great potentials to be applied as anticancer agents.

AgNPs can be produced using physical and chemical methods. These methods are carried out by using hazardous chemicals such as sodium borohydride, potassium bitartrate, methoxypolyethylene glycol and hydrazine which are responsible to reduce their sizes into nanoscale particles. However, these chemicals are highly toxic to human (Sukumaran and Eldho, 2012). According to Hazardous Substance Fact Sheet issued by New Jersey Department of Health and Senior Services, sodium borohydride can cause acute irritation and burn to skin, eyes, nose, throat and lungs. It can also cause severe shortness of breath and affects the nervous system for long term exposure. Moreover, they are not environmental friendly and expensive (Prabhu et al., 2014). Therefore, green synthesis of AgNPs using bacteria, fungi or plants has gained attention and focus of research. Amongst these methods, plant-synthesized AgNPs have been shown to be quicker in reaction as compared to microbes (Sukumaran and Eldho, 2012). Moreover, these methods are less toxic, cost effective and environmental friendly (Ghassan et al., 2013).

As for a preliminary study, AgNPs synthesised by *Catharanthus roseus* aqueous extract were used to evaluate its *in vitro* anticancer activity towards two models of human cancer cells which were Jurkat (leukemic T-cells) and HT29 (colorectal carcinoma) cancer cell lines. Previous study done by Nor Hazwani et al. (2010) has shown that *C. roseus* aqueous has significant inhibitory effects on Jurkat cell. Therefore, a comparison between the cytotoxic effects of *C. roseus* aqueous extract and *C. roseus*-AgNPs on similar cell line should be evaluated. Previous studies also have reported on

the effects of silver nanoparticles on HT29 cells, but to the best of our knowledge, there was no report the on AgNPs synthesised by *C. roseus* being tested on HT29 (Devi and Bhimba, 2012, Sanpui et al., 2011, Devi and Bhimba, 2013, Arunachalam et al., 2015). The basic research and knowledge about their effects on specific cells are important to provide useful information in developing an improved quality and effectiveness of anticancer treatment.

1.2 Research Objectives

The main objective of this study was to evaluate the anticancer activity of *C. roseus*-AgNPs on Jurkat and HT29 cancer cell lines. The specific objectives are as follow:

1. To examine the effects of *C. roseus*-AgNPs on the proliferation of Jurkat and HT29 cells
2. To analyse detection of early and late apoptosis mechanism of Jurkat and HT29 cells treated with *C. roseus*-AgNPs
3. To analyse the cell cycle of Jurkat and HT29 cells treated with *C. roseus*-AgNPs

CHAPTER 2

LITERATURE REVIEW

2.1. Cancer Statistic

Cancer is among the most prevalent disease and one of the main killers worldwide. In a cancer statistics provided by the National Cancer Registry (NCR) Malaysia, the highest reported cancer cases were among Chinese with 50.66 percent, followed by Malays and Indians which were 42.93 percent and 6.41 percent, respectively. This data also showed that women dominated the percentage by 10.8 percent higher compared to men among 18,219 reported cases of cancer (Zainal and Nor Saleha, 2011). According to the World Health Organization (WHO), 14.1 million of new cancer cases were estimated being reported to occur worldwide and the number were expected to increase by 2030. From 100,000 populations, 205.4 cancer occurrences were reported in men and 165.3 in women in 2012. Among all the cancer death reported, lung, breast, bowel and prostate cancer were recorded as the most common causes in worldwide (International Agency for Research on Cancer, 2012, World Health Organization, 2014, Torre et al., 2015).

Leukaemia was the seventh most prevalent cancer among adult age and the most common happened in children. National Cancer Registry of (NCR) Malaysia has reported that leukaemia covers 4.1 percent from 741 cancer cases in 2007 where 490 (66.1%) cases were on 50 years and above patients and the rest that constitute 251 (33.9%) were on children in the range age of 0-14 years old. From this statistic, male patient were higher in percentage compared to female. From the 741 cases, 266 (35.9%) cases reported on adult men and 224 (30.2%) cases were on adult women. Male

children constitute 153 (20.6%) cases followed by female children that are 98 (13.2%) cases. In term of races population, Malays dominate the percentage with 425 cases followed by Indians with 259 cases and the least was Chinese with 57 cases (Zainal and Nor Saleha, 2011).

National Cancer Registry (NCR) Malaysia reported that colorectal cancer is the second most common in Peninsular Malaysia after breast cancer. For the male population, this cancer was the second most prevalent after trachea, bronchus and lung cancer while for female the second after the breast cancer. Colorectal cancer represents 12.3 percent among 18,219 cases registered to NCR in 2007 (Zainal and Nor Saleha, 2011). In the another annual report by National Cancer Patient Registry-Colorectal Cancer that collect the data from October 2007-2008, 60 percent of the total 622 patients were male and the rest were female. From the total registered cases of colorectal cancer, the mean age calculated were 61 years old from the range of 15 to 95 years old of overall age of patients. However, the majority of the patients which constitute 81 percent were aged 50 years and above (Muhammad Radzi and Wendy, 2010).

2.2 Cancer Treatment

Conventional treatments that were used to treat or fight cancer include surgery, chemotherapies and radiotherapies, immunotherapy, targeted therapy, transplantation, cryosurgery, hyperthermia and photodynamic therapy (Torre et al., 2015). However, these conventional therapies were known having many side effects. For example, chemotherapies were not only kills the cancerous cell but also kill healthy dividing cells. This is due to its non-specific action, high toxicity effects and ability to enhance

the development of resistance of the cancerous cells toward the drugs after long-term treatment (Takeuchi and Alison, 2008). Other adverse effects of chemotherapies include thrombocytopenia, neutropenia and anaemia that can cause bleeding, infections, fluid retention, diarrhoea and urinary, kidney and bladder changes in worst case patients (International Agency for Research on Cancer, 2012, World Health Organization, 2014, Torre et al., 2015).

Radiotherapy has the capacity to cause shrinkage and damage of the tumour in a short period of time and that makes it as another common conventional treatment for cancer. However, like chemotherapy, the action of radiotherapy is not specific and can cause toxicity effects to the patients. Despite killing the cancerous tissues, it also can cause inhibition in healthy cells and induce resistance and mutation upon long-term exposure (Tolentino et al., 2011). In addition, the ionizing radiation of the radiotherapy can also induce damages or changes in oral mucosa, skin and salivary glands in normal tissues of nearby area. Furthermore, older patients and children usually have undesirable significant side effects and life threatening effects after treatment (Tolentino et al., 2011, Otmani, 2007).

Another therapy that is used in cancer treatment is immunotherapy which used monoclonal antibody (mAb) or cytokine to specifically target the cancerous cells or boosting the immune system to fight against tumour. However, immunotherapy produces different response in different patients and the treatment also takes longer time that is about 3 to 5 years thus makes it expensive. Other than that, the effectiveness of the treatment also may reduced as the target tumour or fields changed. The side effect of immunotherapy is it can induce severe, life threatening, anaphylactic reactions

(Kirschner and Panetta, 1998). Immunotherapy was commonly used in late stage cancer treatment such as after radiotherapy, surgery or chemotherapy where the immune system of the patients is weakened due to the earlier conventional treatment (Jahrsdörfer and Weiner, 2008).

Due to the limitations of conventional treatment, many studies have been done involving nanotechnology and nanomaterials especially in nanoparticles to overcome the problems. One of the examples was the use of nano sized particles that has been widely used in drug delivery system for chemotherapeutic agents against cancer cells (Mirkin et al., 2011).

2.3 Nanotechnology

Nanotechnology is the development of technology in creating nanoscale devices, systems and materials that sizes range from 1-100 nm. These nanoscale products are unique in terms of physicochemical properties compared to their macroscale size (Cristina et al., 2011). Nanotechnology that is applied in the field of life sciences technology or biotechnology creates the term nanobiotechnology. Examples of applications of nanobiotechnology are nanosurgery, nanolasers for cellular surgery and nano robots. Nanobiomedicine is also one of the applications of nanobiotechnology that specifically relates with medicine. Nanobiomedicine includes the use of nanodevices for diagnosing patients, nanocarrier drug delivery system and also nanopharmaceutical (Jain, 2010). Other than that, production of inert metallic nanoparticles that has potential in therapeutic application like silver, platinum and gold also includes in the application of nanomedicine (Jayachandra et al., 2014).

Another most important branch of nanomedicine is the nanooncology that relates with tumor management. Some of the examples of nanooncology applications are in the diagnosis of cancer using quantum dots, detecting cancer cell using gold nanoparticles conjugated with monoclonal antibody, nanobiosensors to detect multiple protein biomarkers of cancer, magnetic nanoparticles to attach and detect the floating cancer cell in blood circulation and target delivery of drugs to cancerous site by nanocarrier (Jain, 2010).

Nanoparticles or nano treatment is emerging as a new approach in cancer treatment because of their unique physical and chemical characteristics. They are very small in size thus makes them have large surface area to volume ratio. This property enables nanoparticles to pass through the cellular barrier easily and also able to efficiently carry conjugated compounds such as drugs, nucleic acids, antibody, proteins and probes (Thakor and Gambhir, 2013). Moreover, the nano treatment also more favoured because of their characteristics such as longer shelf life, more stable, specific and only needed in low dosage frequency compared to conventional treatment (Jacob et al., 2013). The examples of nanoparticles derived drugs in the different phase of clinical development were illustrate in Table 2.1.

2.3.1 Biomedical Application of Nanomaterials

Nanomaterials were already being used widely in medical practice such as in invasive imaging techniques, targeted drug delivery systems and in the development of engineered organs. Table 2.2 shows that various type and structure of nanomaterials that has been used in biomedical applications. An example, nanoscaled drug delivery

systems such as liposomes and drug-conjugated nanoparticles were used for cancer treatment in drug chemotherapy (Barkalina et al., 2014, Ulatowska-Jarza et al., 2011).

Table 2.1 Nanoparticle derived drugs in the different phase of clinical development. Table taken from Bhattacharyya et al. (2011)

Products	Combidex (Ferumoxtran-10)	Biovant	CYT-6091 (Aurimmune)	Bio-conjugated nanoparticles	Abraxane
Type of nano materials	Iron oxide nanoparticles	Nano sized calcium phosphate	TNF-α bound PEGlyated colloidal gold particle	Luminescent quantum dots	Albumin-bound paclitaxel particles
Size (nm)	17-20	NAD	33	10-15	130
Application	Tumor imaging	Vaccine component	Solid tumors	Cancer imaging	NSC lung cancer, breast cancer, others
Route of administration	IV	IM	IV	Subc	IV
Phase	NDA field	Phase I completed	Phase I	Preclinical	Approved
Company	Advanced Magnetics	BioSante Phamaceutical	Cytlmmune Sciences	Emory-Georgia tech nano-technology centre	Abraxis Oncology

The drugs that were conjugated with biocompatible polymers were found to reduce the systemic toxicities and inducing the possible reformulation of existing drugs. The example was Polyethylene glycol (PEG) coated liposomes that conjugated with anthracycline doxorubicin that shows an effective effect against doxorubicin-resistant colorectal cancer. In order to encourage sufficient mechanical strength, expanding cellular growth and maximizing the potential of osseointegration, nanoparticles-conjugated polymer are used to induce the reinforcement of the structure (Loizidou and Seifalian, 2010, Nie et al., 2007).

Furthermore, the ability of bacteria to develop resistance against antibiotics after extended time encourages the development of new strategies in order to cope with the implant related infections. Therefore, silver that were known to have antibacterial activities were used in silver-based biomaterial to overcome this limitations. The characteristic makes the silver-based materials to be used worldwide to protect the inner and outer surfaces and the proximity of a medical device to protect the implant from infection (Eckhardt et al., 2013).

Table 2.2 Biomedical applications of nanomaterials. Table was taken from Barkalina et al. (2014)

Class	Subclass	Material	Structure	Description
Organic	Lipid	Phospholipids	Liposome	Enclosed nanospheres comprised of a phospholipid bilayer
			Micelles	Enclosed nanospheres comprised of a phospholipid monolayer
		Solid lipid	Solid lipid nanoparticles	Nanospheres comprised of the lipid core stabilized by surfactants and/or polymers
	Polymers	Poly-L-lactide-co-glycolide (PLGA)	Nanoparticles	Variously shaped structure with all three physical dimensions on the nanoscale (< 100 nm)
		Poly-L-lactic acid (PLA)		
		Chitosan		
		Gelatine		
		Polyamidoamine (PAMAM)	Dendrimers	Spherical nanomolecules consisting of the central core and sequential layers of branching groups
		Polypropyleneimine (PPI)		
Inorganic	Noble metals	Gold	Nanoparticles	Various shaped structures with all three physical dimensions on the nanoscale (< 100 nm)
		Silver		
		Platinum		
	Oxides	Magnetic and superpara magnetic iron	Nanoparticles	Various shaped structures with all three physical dimensions on the

	oxides		nanoscale (< 100 nm)
Semiconductors	Cadmium Selenium Tellurium Indium	Quantum dots	Semiconductor nanocrustals with optical properties
Carbon-based	Carbon	Fullerences	Hollow nanospheres, comprised of carbon atoms, forming cage-like structure
		Nanotubes	Cylindrical structures with two of three physical dimensions on the nanoscale (< 100 nm)
Other	Mesoporous silica	Nanoparticles	Various shaped structures with all three dimensions on the nanoscale (<100 nm) and mesoporous architecture (pore diameter: 2-50 nm)

2.3.2 Silver Nanoparticles (AgNPs)

Silver is a type of inert inorganic metal element that exists naturally in environment. Silver ions are the product of ionisation of silver metal that can kill microbes. Silver was found to have medicinal properties since hundred years ago and being used as antibacterial and antimicrobial agent long before the discovery of microbial agent during the Germ Theory of Disease (Barillo and Marx, 2014). The earliest usage of silver is as silver vessels or in the form of coins to store and disinfect water. This was followed by other medicinal application such as antiseptics and use in burn and wound care (Barillo and Marx, 2014, Gao et al., 2015).

The development of nanotechnology nowadays makes the silver metal being able to be produced in nanoscale particles with sizes less than 100 nm. The nanoparticles form of silver enhanced in physical, chemical and biological properties when compared to its bulky form (Muthu et al., 2010). AgNPs are more favourable due

to their characteristics such as chemically stable, reliable, cheap in production, have catalytic properties in chemical reaction, good conductivity, anti-inflammatory, and antimicrobial activity against various infectious bacteria includes *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Saranyaadevi et al., 2014, Hunt et al., 2014). They kill the microbes by disrupting the respiratory function and permeability of cell and destruct the DNA to inhibit the growth of the microbes (Mohammed, 2015).

Thus, the properties make them not only being applied in many consumers products, food production or cosmetics but also in medical and biomedical field including in wound dressing, as antiseptics, dental fillers and as a coating materials on medical devices to prevent biofilm production by bacteria (Garvita et al., 2014). Silver nanoparticles were proven having high toxicity towards many microorganisms. However, they were found to be less or non toxic towards human and other mammalian cells at low concentration (Jacob et al., 2013, Garvita et al., 2014).

In recent findings, AgNPs were recorded to have inhibitory effect on the proliferation against several cancers *in vitro*. This include breast cancer MCF-7 cell lines (Khatiravan et al., 2014, Gnanasekar et al., 2014), Dalton's lymphoma ascites (DLA) cell lines (Muthu et al., 2010), human lung carcinoma A549 cell lines (Renu et al., 2013, Balaji et al., 2014), human cervical carcinoma cell lines (Karunamoorthy et al., 2014, Panchanathan et al., 2013), colo205 cell line (Garvita et al., 2014) and human glioblastoma cell lines (AshaRani et al., 2009).

The nanoscale size makes them to become chemically active and produced reactive oxygen species (ROS) and free radicals that can cause inflammation and damage to the cell. Studies proved that silver nanoparticles can cause oxidative stress and damage on genetic material when they get into sarcoma cells, primary neural cells and carcinoma cells by inducing the production of reactive oxygen species. Other than that, silver nanoparticles also proved to induce apoptosis on cells by increasing the tumor suppressor gene expression (Rajanahalli et al., 2015).

There are several ways to produced silver nanoparticles. In early technology, silver nanoaparticles were produced through physical and chemical method (Renu et al., 2013). The disadvantage of physical method is the low yield production while for chemical method; it is also less preferred because it requires the usage of harmful and toxic chemical such as sodium borohydride and citrate to reduce the silver ions to nanoparticles form (Sangiliyandi et al., 2013b). The by-products from this method also are harmful to the environment. In addition, the methods also costly and need a specific condition to produce the nanoparticles (Rajan et al., 2015).

Nowadays, green method of production of silver nanoparticles gains more attention over the early method. Green method includes the use of bacteria, fungi and plant extracts as the reducing agent for silver ions (Khatiravan et al., 2014). In general, green method are more preferable because of they are safer to the environment, need less cost of production, not require specific condition and can produce in large quantity (Garvita et al., 2014). However, above all the green methods, plant aqueous extract are the one that is most preferable because it not require any cell culture method that can have the risk of contamination occurring (Khatiravan et al., 2014). Moreover, the waste

product of microorganisms needs to be properly managed because it may harmful to the environment depending on the types of microorganisms used. The production using plant aqueous extract also can be very simple and fast because plants contains high reduction potential compared to other two methods. The alkaloids and phytochemicals that present in the plant extract also can give the effect of stability towards the nanoparticles that were produced (Rajan et al., 2015).

There are many reports that show the production of silver nanoparticles from plant extract including *Melia dubia* (Khatiravan et al., 2014), *Acorous calamus* (Jayachandra et al., 2014), *Moringa olifera* (Karunamoorthy et al., 2014), *Ficus religiosa* (Jacob et al., 2013), *Eucalyptus chapmaniana* (Ghassan et al., 2013), *Sesbania grandiflora* (Jeyaraj et al., 2013), *Eucalyptus globulus*, *Ziziphus spina-christi*, *Eucalyptus angophoroides*, *Camellia sinensi*, *Eucalyptus camaldulensis* (Mohammed, 2015), *Origanum vulgare* (Renu et al., 2013) and *Catharanthus roseus* (Mukunthan et al., 2011). Almost all of these biosynthesized silver nanoparticles exhibit anticancer activity towards various cancer cell lines.

2.4 *Catharanthus roseus* (L) G. Don

Catharanthus roseus (L) G. Don or also known as *Vinca rosea* as shown in Figure 2.1 is an important medicinal plant from Apocynaceae family that wildly grows in tropical and subtropical climate (Dutta et al., 2005). This Madagascar periwinkle is a type of flowering herbaceous plant that usually grows upright or decumbent, up to one metre height and secretes latex that produces pungent odour when damaged. Despite being used as ornamental plant, *Catharantus roseus* (*C. roseus*) also were known as an important therapeutic plant that was used traditionally to cure various diseases including

diabetes, leukaemia, insect bites, dysentery, and skin infection (Nejat et al., 2015, Pankaj et al., 2008).

Figure 2.1: *Catharanthus roseus* plant. Figure is taken from Nejat et al. (2015)

C. roseus produces more than 120 terpenoids indole alkaloids (TIAs) including vinblastine, vincristine, ajmalicine, catharantine, vincoline, vincubine, vinorelbine, vindesine and serpentine. Those alkaloids that accumulated in different parts of the plant have important pharmacological value such as antitumor, sedative and antihypertensive (Rischer et al., 2006). Vinblastine, vincristine and vinorelbine were already being approved as therapeutic substances in United States while several other have been undergo clinical trials (Moudi et al., 2013). The most valuable alkaloids of *C. roseus* are the vinblastine and vincristine as they have the anticancer properties. These alkaloids are accumulated in the leaves or aerial parts of the plant and being produced in low amount by the plants (Rischer et al., 2006, Dutta et al., 2005). They are the first natural drugs that were used in the treatment of cancer and commonly been used together with the chemotherapy drugs in cancer therapy (Maria et al., 2008). Vinblastine

was approved as a part of treatment of testicular carcinoma, Hodgkin and non-Hodgkin lymphoma, breast cancer and tumour of germ cell. Vincristine was used as treatment for acute lymphoblastic leukemia, Wilm's tumor in children, neuroblastoma, rhabdomyosarcoma, Hodgkin lymphoma and other lymphomas (Moudi et al., 2013).

The mechanism of actions of these alkaloids is by interacting with tubulin and disrupting the mitotic spindle apparatus of the microtubules. This interaction cause blocking in mitosis process and lead the cancer cell to metaphase arrest (Chun-Hua Wang et al., 2012). Since these alkaloids interact and disrupt the microtubules, they can be highly toxic not only to cancer cell but also to non-malignant cell as microtubules involve in many cell functions (Moudi et al., 2013). Therefore, many side effects being reported that consist of vomiting, toxicity of nervous system and white blood cells, suppression of the activity of bone marrow, dyspenia, kidney problems, damage of liver cell, mouth ulcer, constipation, nausea, fever, urinary retention, abdominal cramps, and hair loss or alopecia. Hence, the usage of these alkaloids should be under controlled and correct dosage must be prescribed in order to minimize the side effects (Moudi et al., 2013, Nejat et al., 2015).

CHAPTER 3

MATERIALS AND METDODS

3.1 Materials

All autoclavable materials including glassware and plastic ware were sterilised and autoclaved at 121 °C for 30 minutes at a pressure of 100 kPA prior to use.

3.2 Methods

3.2.1 Preparation of *C. roseus* aqueous extract

The method of extraction was performed as according to Nor Hazwani et al. (2010). *C. roseus* plants were collected from Teluk Air Tawar, Butterworth, Penang. The plant specimen was sent for identification at Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia (USM). The voucher specimen identification of the plant is 10933. The fresh leaves were washed and dried in 40 ˚C oven. The dried leaves were grounded into powder and 50 gm was dissolved in 1 L distilled water. The mixture was incubated for 24 hours in water bath shaker at 40 ^{o}C. The mixture was centrifuged for 15 minutes at 860 × g before filtered by using Whatmann filter paper No 1 (Sartorius, Germany) . Then, the extract was freeze dried in freeze dryer (FDU-1200, Eyela, USA) and was kept in -20 ^{o}C for storage and ready to be used.

3.2.2 Synthesis of *C. roseus*-AgNPs.

The AgNPs has been synthesised, optimised and characterised previously. Five mL of 10% *C. roseus* aqueous extract (w/v) was dissolved with 45mL of 5 mM silver nitrate ($AgNO_3$) solution. The solution was left at room temperature for 24 hours until a brown-yellow solution was formed. The brown-yellow solution was the sign of the presence of AgNPs. The solution was centrifuged at 7700 × g for 15 min. The

supernatant was discarded and the pellets were washed with distilled water two times before dried in oven for 48 hours at 40 ^{o}C. The characteristics of *C. roseus*-AgNPs that were used in this study were summarized as below:

Characterizatios	***C. roseus*-AgNPs**
Synthesis	10% of *C. roseus* aqueous extracts in 5 mM of $AgNO_3$
Surface Plasmon (UV-Vis spectroscopy)	500 nm
Transmission Electron (TEM)	Shape : Spherical and uniform Size : 20 nm to 50 nm Diameter : 30 nm
Particle Size and Zeta Potential Measurement	Mean particle size: 167 nm Zeta potential : 0.0295 mV
X-ray Diffraction (XRD) analysis	Spectrum 2θ values : 38.12º, 44.31º, 64.45º and 77.41º Plane : 111, 200, 220 and 311 Structure : Crystals in nature

3.2.3 Preparation of complete media

Jurkat and HT29 cells were cultured in a complete Roswell Park Memorial Institute-1640 (RPMI-1640) (Nacalai Tesque, Japan) medium. Two gram sodium bicarbonate (Sigma, USA) was weighted and mixed well with 900 mL of Ultra-Pure Water in a conical flask. Ultra-Pure Water was added until up to 1 L after the pH of the solution was adjusted to pH 7.1. The medium was filtered sterilised by using 0.22 µm disposable filter system that connected with vacuum pump in the Class II Biosafety Cabinet. Five mL of the prepared medium was put into 25 cm^2 flask and incubated for 24 hours in the incubator (Shellab, USA) at 37 ^{o}C with 5% CO_2. The sterility of the prepared medium was checked before proceed to produce complete medium. The medium were added with 10% heat-inactivated fetal bovine serum (v/v) (Nacalai Tesque, Japan), 1% of 100 units/mL penicillin-streptomycin (v/v) (Nacalai Tesque, Japan) and 1% of 200 mM-L-Glutamine Stock Solution (v/v) (Nacalai Tesque, Japan) as supplements to make it a

complete medium. Prior to use, the fetal bovine serum was heat-inactivated in 56 ^{o}C water bath for 30 min.

3.2.4 Freezing cells

The confluency of Jurkat and HT29 cells were checked under inverted microscope (Carl-Zeiss, USA). The cells were taken out from the 25 cm^2 culture flask and transferred into a 15 mL centrifuge tube (BD Biosciences, USA) after reaching 70 to 80% confluency. HT29 cells were detached first by using 1mL 2.5 g/l-Trypsin/1mmol/l-EDTA solution (Nacalai Tesque, Japan). The cells were centrifuged at 500 × g for 5 minutes. The supernatant was removed and the pellet was resuspend with ice cold freezing medium which contains the mixture of 10% dimethyl sulphoxide (DMSO) (v/v) and 90% of fetal bovine serum (v/v). The mixture was aliquot into sterile 1.8 mL cryotube vials (Nunc, Denmark) and was put in a container before stored in -80 ^{o}C freezer for overnight. After that, the cells were transferred to the liquid nitrogen storage tank.

3.2.5 Thawing cells

Frozen cells from liquid nitrogen tank were immediately immersed in a water bath at 37 ^{o}C. The semi-fluid cells suspension were diluted in pre-warmed complete RPMI-1640 growth medium (Nacalai Tesque, Japan) and centrifuged at 500 × g for 10 minutes. The DMSO containing supernatant was then discarded and the pellet was resuspend with pre-warmed complete RPMI-1640 growth medium before transferred into 25 cm^2 tissue culture flask. The cells suspension was then incubated in the incubator (Shellab, USA) at 37 ^{o}C with 5% CO_2. Cells were routinely checked under inverted microscope (Carl-

Zeiss, USA) to determine the confluency. The cells were subculture when the cells reached 70 to 80% confluence.

3.2.6 Subculturing cells

Jurkat and HT29 cell lines were obtained from American Type Culture Collection (ATCC). The cells was maintained in complete medium of RPMI-1640 containing 10% heat-inactivated fetal bovine serum (v/v), 1% of 100 units/mL penicillin-steptomycin (v/v) and 1% of L-glutamine (v/v). Cells were routinely observed under inverted microscope to make sure that no signs of contaminations of bacteria or fungi. The culture medium was change in every 3 days to maintain the good condition for the cells. Jurkat cells were taken out from the 25 cm^2 culture flask and transferred into a 15 mL centrifuge tube (BD Biosciences, USA). HT29 cells were detached first by using 1mL 2.5g/l-Trypsin/1mmol/l-EDTA solution. The cells were centrifuged at 500 × g for 5 minutes. The supernatant was discarded and the pellet was resuspend with new complete RPMI-1640 medium and transferred back into the larger culture flask. Then, the flask containing cells were incubated in the incubator at 37 ^oC with 5% CO_2. Cells were grown until reached 70 to 80% confluency before used in experiments.

3.2.7 MTS Assay

Cell proliferation was assessed by using Cell Titer 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega, USA) which composed of [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carbonxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine metho sulphate; MTS] and (phenazine methosulfate; PMS). The procedures were carried out as stated in the protocol of the kit. Jurkat and HT29 cells were seeded in a 96-well plate at a density of 4×10^5 cell/mL and 1×10^5 cell/mL, respectively. Cells were treated with

C. roseus-AgNPs and *C. roseus* aqueous extract in serial dilution manner which were 1.96 µg/ml, 3.91 µg/ml, 7.82 µg/ml, 15.63 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml in 96-well plates. The cells were incubated in an incubator for 24, 48 and 72 hours. Cells also were treated with silver nitrate ($AgNO_3$) of concentration 1000 µg/ml. Camptothecin at a concentration of 1.25 µg/ml was used as a positive control and the untreated cells were used as negative control. Each of the sample size was in triplicate. Following the incubation time interval, the cultured cells in each well were added with 20 µL of mixture of MTS/PMS solution with the ratio of 20:1. The cells were then incubated for 1 to 4 hours at 37 ^{o}C in a humidified 5% CO_2 incubator. The 96-well plates were analysed at 490 nm using ELISA plate reader (Bio Tek, USA). The half minimal inhibitory concentration (IC_{50}) values were determined from the graph to get the treatment concentration that caused 50% cell death. The cell viability (%) was calculated as follows:

$$\text{Percentage Cell Viability (\%)} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated (control) cells}} \times 100$$

Where, OD is the optical density.

3.2.8 Annexin V-FITC/Propidium Iodide Assay

The externalised phosphatidylserine phospholipid was examined by using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, USA). The protocol used was carried out as stated in the manual. Cells at a concentration of 4×10^5 cells/mL for Jurkat and 5×10^5 cells/mL for HT29 were treated with three different concentrations (5 µg/mL, 10 µg/mL, 15 µg/mL) of either *C. roseus*-AgNPs or *C. roseus* aqueous extract and 1000 µg/mL of $AgNO_3$ solution for 6, 24, 48 and 72 hours. Camptothecin with concentration of 1.25 µg/mL was used as a positive control and untreated cells as negative control.

The cells were harvested and washed twice with 1X cold phosphate buffered saline (PBS) (Sigma-Aldrich, USA). Then, the cells were centrifuged at 500 × g for 5 minutes before resuspend with 100 µL of 1X binding buffer (0.1 M Hepes (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. Approximately 100 µL cell solutions were transferred into 5mL round bottom culture tube (BD Biosciences, USA). Five µL of Annexin V-FITC and 5 µL of Propidium Iodide (PI) were added to stain the cells. The cells were gently mixed by using vortex and incubated at room temperature in the dark for 15 min before added with 1X binding buffer (400 µL) to stop the reaction. The stained cells were analyzed by FacsCalibur flow cytometry (BD FACSCalibur Flow cytometry, US) at 10,000 events.

3.2.9 Cell Cycle Analysis

Cell cycle arrested were analyse using Cycle Test^{TM} Plus DNA Reagent Kit and DNA QC Particles (Becton Dickinson, USA) and the proceduresl was carried out following the manufacturers' recommendation. Jurkat and HT29 cells at concentration of 4×10^5 cells/mL and 5×10^5 cells/mL, respectively were treated with 10 µg/mL *C. roseus*-AgNPs or *C. roseus* aqueous extract and 1000 µg/mL of $AgNO_3$ solution. The cells were incubated in the incubator at 37 ^oC in 5% CO_2 humidified atmosphere for 24, 48 and 72 hours. Untreated cells were used as negative control and camptothecin with concentration of 1.25 µg/mL was used as positive control. After each incubation hour, cells were harvested and washed with buffer solution three times. The cells were counted and the concentration was adjusted to 5×10^5 cells. The cell suspensions were centrifuge at 400 × g for 5 minutes. The supernatant was discarded and 250 µL of Solution A (trypsin buffer) was added and mixed gently before being left for 10 minutes at room temperature. 200 µL of Solution B (trypsin inhibitor and RNase buffer) were